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Queen Execution, Diploid Males, and Selection For and Against Polyandry in the Brazilian Stingless Bee *Scaptotrigona depilis*

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ABSTRACT: Female mating frequency varies. Determining the causes of this variation is an active research area. We tested the hypothesis that in stingless bees, Meliponini, single mating is due to the execution of queens that make a matched mating at the complementary sex determination locus and have diploid male offspring. We studied the Brazilian species *Scaptotrigona depilis*. We made up 70 test colonies so that 50% (single matched mating), 25% (double mating), 12.5% (quadruple mating), or 0% (single nonmatched mating) of the emerging brood were diploid males. Queen execution following diploid male emergence was equal and high in colonies producing 50% (77% executed) and 25% (75%) diploid males versus equal and low in colonies producing 12.5% (7%) and 0% (0%) diploid males. These results show that queens that mate with two males with similar paternity suffer an increased chance of being executed, which selects against double mating. However, double mating with unequal paternity (e.g., 25:75), which occasionally occurs in *S. depilis*, is selectively neutral. Single mating and double mating with unequal paternity form one adaptive peak. The results show a second adaptive peak at quadruple mating. However, this is inaccessible via gradual evolutionary change in a selective landscape with reduced fitness at double mating.

Keywords: diploid male load, queen mating frequency, mating system evolution, twin adaptive peaks.

Introduction

The number of males that females mate with ranges from monandry to promiscuity. The underlying reasons for this variation and, in particular, for the evolution of polyandry is an important area of research (Pizzari and Wedell 2013; Taylor et al. 2014). In the eusocial Hymenoptera, this research has additional significance, as the number of males that queens mate with affects kinship (Hamilton 1964), which itself has many effects on social evolution, including the evolution of eusociality (Hughes et al. 2008) and the structures and outcomes of intracolony reproductive conflicts (Ratnieks et al. 2006).

In eusocial Hymenoptera, polyandry has evolved multiple times (Hughes et al. 2008). Queens of different species mate with 1–40 or more males at the start of their adult lives and do not remate (Boomsma and Ratnieks 1996; Strassmann 2001). There are many potential adaptive reasons for variation in queen mating frequency and the evolution of polyandry (Crozier and Page 1985; Boomsma and Ratnieks 1996; Palmer and Oldroyd 2000; Crozier and Fjerdingstad 2001). In the bumble bee *Bombus terrestris*, mating may be constrained by a male-produced mating plug that prevents queen remating (Baer et al. 2001). However, most hypotheses underlying variation in queen mating invoke some effect arising from the fact that queens live in a colony, that the workers are her daughters, and that queen fitness is affected by social living (Crozier and Page 1985; Palmer and Oldroyd 2000). A queen's fitness is greatly affected by the success of her colony. In honey bee *Apis mellifera* colonies, polyandry may reduce the impact of diseases (Sherman et al. 1988; Seeley and Tarpy 2007; Mattila and Seeley 2007) and improve work performance (Mattila and Seeley 2007) and homeostasis (Jones et al. 2004; Oldroyd and Fewell 2007). In the wood ant *Formica truncorum*, polyandry enhances queen fitness not by increasing colony performance but by causing the workers to allocate reproduction more to rearing males and less to rearing young queens, which increases the mother queen's inclusive fitness (Sundström and Ratnieks 1998).

The stingless bees, Meliponini, comprise several hundred eusocial species living worldwide in the tropics and southern subtropics (Camargo and Pedro 2013). As in honey bees (*Apis*), there is a strong queen-worker reproductive division of labor and morphological differentiation, with pe-

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rennial colonies and the ability to replace a failing or dead queen (Michener 1974). In contrast to *Apis*, in which queens of all species are highly polyandrous (Oldroyd et al. 1997; Palmer and Oldroyd 2000; Tarpy et al. 2004), stingless bee queens are typically mated to one male (Palmer et al. 2001; Vollet-Neto et al. 2018).

One hypothesized reason for monandry in stingless bees involves costs arising from the production of diploid males (Ratnieks 1990). In many Hymenoptera, sex is determined by a genetic system known as complementary sex determination (CSD; Whiting 1943; Cook and Crozier 1995; Heimpel and Boer 2008). Individuals that are heterozygous at the CSD locus are female. Homozygotes and hemizygotes (haploids) are male. When a female hymenopteran in a species with CSD makes a matched mating with a male sharing one of her two (as females are heterozygous) alleles at the CSD locus, then 50% of her diploid offspring will be males (e.g., AB [female] × A [male] → 50% AB [diploid females] + 50% AA [diploid males]). By contrast, when a female makes an unmatched mating, then all of her diploid offspring are female (e.g., AB [female] × C [male] → 50% AB [diploid females] + 50% AC [diploid females]). Matched matings are relatively rare in stingless bees (only 5%–10%; Vollet-Neto et al. 2018) because frequency-dependent selection favors rare alleles at the CSD locus (Gloag et al. 2016) and so generates and maintains high allelic diversity, although allelic diversity can be low in small populations due to drift (Yokoyama and Nei 1979; Cook and Crozier 1995; Zayed and Packer 2001).

Stingless bee queens that have made a matched mating are replaced, presumably executed by the colony's workers, soon after their first offspring, including adult diploid males, emerge from their cells (Camargo 1979; Alves et al. 2011; Vollet-Neto et al. 2017). Replacement is presumably because a colony cannot function adequately or even survive when half the offspring that would normally become adult workers instead become adult diploid males, who do no work yet need resources to rear. As a result, both the workers and the executed queen would benefit from replacing a queen that has made a matched mating by her daughter, who is unlikely to make a matched mating, even though this will result in a reduction in relatedness to future males and queens reared.

One hypothesized consequence of queen execution in stingless bees is selection for monandry by queens (Ratnieks 1990). The underlying logic depends on the relationship between a queen's execution probability and the proportion of diploid males in her offspring. In particular, double mating increases the probability of making a matched mating, which is approximately doubled (see box 1), but reduces the proportion of diploid males from 50% to 25%, assuming equal paternity and that only one male is matched. If 25% diploid male offspring is enough to trigger execution, then double mating will be selected against, as overall execution probability will increase. However, if 25% diploid males does not trigger execution or reduces execution probability to less than half that of 50% diploid males, then double mating will be selected for in queens, as the overall probability of execution is reduced.

We tested the increased probability of execution hypothesis in the Brazilian stingless bee *Scaptotrigona depilis* (Apidae, Meliponini), a species known to execute queens that make a matched mating (Vollet-Neto et al. 2017) and to have queens that mostly are mated to one male but sometimes to two males with highly unequal paternity (Paxton et al. 2003). We transferred brood combs from colonies with queens producing 0% and 50% diploid males to create (and quantify queen elimination in) test colonies in which 25% of diploid offspring were males, thereby simulating the outcome of mating with two males with equal paternity, one a matched mating and the other unmatched. This enabled us to test the hypothesis that single mating is selectively favored over double mating with equal paternity due to queen execution. In addition, queen elimination was also determined in colonies producing 12.5% diploid males, equivalent to mating with four males with equal paternity with one being a matched mating or with two males with unequal paternity with the minority male being a matched mating. This enabled us to test two additional hypotheses: (1) that mating with four males is selectively favored over single mating and (2) that double mating with unequal paternity is selectively neutral in comparison to single mating. The results support all three hypotheses and indicate that there is a fitness landscape with two peaks (single mating or double mating with unequal paternity; polyandry) separated by a fitness valley (double mating with equal paternity).

Methods

Study Species and Site

The study was carried out using colonies of *Scaptotrigona depilis* kept in outdoor wooden hives (internal cavity measurements, 42 × 28 × 18 [high] cm) at the experimental apiary of the Department of Biology of the University of São Paulo at Ribeirão Preto (USP RP) and at the apiary of PROMIP near the town of Engenheiro Coelho, state of São Paulo, Brazil. The two locations are 150 km apart. *Scaptotrigona depilis* naturally occurs in this region (Camargo and Pedro 2013). Colonies have a single egg-laying queen. Mature colonies have approximately 5,000–10,000 workers.

Obtaining Matched-Mated Queens

Scaptotrigona depilis is common on the USP RP campus. A census over 9 years (http://www.apacame.org.br/mensagemdoce/100/artigo3.htm; database accessed on February 28,

2018) found 109 colonies nesting in hollow trees on the 5.8-km^2 campus. Many more colonies would have been present but undetected. To obtain queens that had made a matched mating, we made a total of 99 removals of the mother queen, using the USP RP hives. We then allowed a replacement daughter queen to take over and start egg laying, which we detected by the presence of newly sealed brood cells. Stingless bees mass provision brood cells, with cell sealing taking place immediately after the queen has laid an egg in the cell (Michener 1974).

We had previously determined that the egg to adult brood period in *S. depilis* is approximately 36 days (Vollet-Neto et al. 2017). Therefore, approximately 30 days after a new queen began egg laying we inspected pupae taken from brood combs to identify colonies in which approximately 50% were male on the basis of their characteristic morphology, including having smaller heads and larger eyes than workers.

From each colony 10 males were collected and checked using DNA microsatellites at three loci to confirm that they were diploid. Males were kept in absolute ethanol until DNA was extracted using the Chelex method. They were then genotyped at three microsatellite loci, T3 (Paxton et al. 1999) as well as Sxant06 and Sxant18 (Duarte et al. 2012). Microsatellite amplification and visualization followed Francisco et al. (2011). We confirmed males as diploid if they were heterozygous at one or more loci (Alves et al. 2011).

In total, eight queens that had made a matched mating were obtained. As *S. depilis* queens have single or close to single paternity (Paxton et al. 2003), this also gave us an estimate of the probability of making a matched mating ($8/99 = 0.0808$). These matched-mated queens were marked by clipping their wings and were introduced into queenless colonies with brood, workers, and food stores. Egg-laying *S. depilis* queens are easily accepted by queenless colonies (Vollet-Neto et al. 2017). To provide an ongoing source of brood with 50% diploid males, we prevented the execution of our matched-mated queens by repeatedly transferring them, at intervals of approximately 1 month, into colonies without diploid males from which we had just removed the queen.

Producing Experimental Colonies with Different Proportions of Diploid Males

To simulate colonies in which the queen has mated with two males with equal paternity, one being a matched mating (i.e., 25% diploid males), we set up test colonies in which half the brood came from a source colony headed by a matched-mated queen (50% diploid males) and half came from a non-matched-mated queen (0% diploid males; fig. 1). By choosing brood source colonies of equal strength, we ensured that there were similar numbers of emerging adult bees from each. In addition, to simulate one matched mating out of four matings with equal paternity or double mating with unequal paternity (25% vs. 75%), we set up test colonies in which approximately 12.5% of the emerging bees were diploid males. We did this in a similar way but with only one-quarter of the transferred brood from the matched-mated queen versus three-quarters from the non-matched-mated queen. To control for the use of 25% and 12.5% diploid males in test colonies with brood from two source colonies, the test colonies producing 50% and 0% diploid males were also given brood from two source colonies, both producing either 50% or 0% diploid males, and received all of the same manipulations.

For each test colony, combs containing pupae were introduced four times at intervals of approximately 18 days. At every brood comb introduction we removed approximately the same amount of older brood combs containing pupae from the test colony. These contained progeny of the test colony's own queen, who had not made a matched mating. Any adult bees on these combs were gently brushed from the combs back into their hive. During the test period, the only brood that gave rise to adult bees within each test colony was the brood that had been transferred into that colony from the two source colonies. This resulted in a period of approximately 70–80 days in which the test colonies had bees emerging from their cells in the designated experimental proportions (0%, 12.5%, 25%, and 50% diploid males). This duration was sufficient, as new queen execution in *S. depilis* generally occurs within approximately 40 days of the start of the emergence from their cells of the first of her adult offspring (Vollet-Neto et al. 2017).

Determining Survival of Queens in Colonies with Different Proportions of Diploid Males

Every test colony had an egg-laying queen, marked by clipping her wings, that we knew had not made a matched mating, as her offspring pupae contained a low proportion of males. (Some haploid males are also reared by colonies.) Previous research has shown that a queen that has made a matched mating is not detected by her mating status but by the adult diploid males in the colony (Vollet-Neto et al. 2017). Thus, a matched-mated queen whose brood is prevented from emerging is not killed (Camargo 1976; Vollet-Neto et al. 2017; this study), and a non-matched-mated queen in a colony given brood with 50% diploid males is usually killed (Vollet-Neto et al. 2018; this study).

To give a consistent and biologically relevant survival assay, the queen in each test colony was monitored by making hive inspections every 15 days until she was missing (presumably having been killed by the workers) or had survived 90 days. We determined that the marked queen was dead when we observed either a new nonmarked egg-laying queen

Box 1: Effect of queen execution on the fitness of queens mated to different numbers of males

A. Single mating versus double mating

This analysis determines the overall probability of queen execution, resulting from diploid male production, for single- versus double-mated queens.

Parameters

- M_i = total execution probability of queens of mating type i.
- p = probability of a matched mating in the population.
- m_x = execution probability of a queen in a colony with proportion x diploid males.

Total execution probabilities

Single mating. Scenario: random mating. If there is a matched mating, then the proportion of diploid males in the colony is 50%. Note that $m_0 = 0$.

$$M_1 = m_0\,(1-p) + m_{0.5}p = m_{0.5}p \quad (\text{as } m_0 = 0). \tag{1}$$

Double mating. Scenario: random mating. The paternities of the two males are x and $(1-x)$. If one of the two males is a matched mating, then the proportion of diploid males is either $(x/2)$ or $(1-x)/2$, with equal probability. If both males are matched matings, then the proportion of diploid males is 0.5.

$$M_2 = m_0\,(1-p)^2 + m_{x/2}\,[p(1-p)] + m_{(1-x)/2}\,[p(1-p)] + m_{0.5}p^2. \tag{2}$$

Queen fitness

For single mating to be favored over double mating, then M_1 must be less than M_2. From equations (1) and (2), this is

$$m_{0.5}p < m_{x/2}[p(1-p)] + m_{(1-x)/2}[p(1-p)] + m_{0.5}p^2, \tag{3}$$

$$m_{0.5}[p(1-p)] < m_{x/2}[p(1-p)] + m_{(1-x)/2}[p(1-p)], \text{ which simplifies to}$$

$$m_{0.5} < m_{x/2} + m_{(1-x)/2}. \tag{4}$$

Inequality (4) shows that the probability of making a matched mating does not affect the result as parameter p cancels out. Single mating is favored when the queen execution probability in a colony producing 50% diploid males is less than the sum of the two queen execution probabilities for a colony with double mating, corresponding to the paternities of the two males (e.g., the majority male and the minority male). When males contribute equal paternity (i.e., $x = (1-x) = 0.5$) then the proportions of diploid males are both 25% (i.e., $x/2 = (1-x)/2 = 0.25$). When this occurs, our data show that inequality (4) is true as $m_{0.5} = 0.77$ and $m_{0.25} = 0.75$ (i.e., $0.77 < 0.75 + 0.75$). When paternity is unequal, then in the limiting case of highly unequal paternities (i.e., $x/2$ approaches 0.5 and $(1-x)/2$ approaches 0), inequality (3) will become the equality $m_{0.5} = m_{0.5}$, showing that double mating with highly unequal paternity is neutral. Our data show that inequality (3) is approximately an equality for $x = 0.75$, as $m_{0.125}$ is low, 0.07. Although we do not have empirical data for $m_{0.375}$, we do have data for $m_{0.5}$ and $m_{0.25}$. These are almost the same numerically, 0.77 and 0.75, and not statistically different ($P = 1.0$), showing that double mating with paternities of 75%:25% is approaching neutrality.

B. Single mating versus polyandry

This analysis considers the effects of queen execution resulting from diploid male production on the fitness of queens mated to a single male versus to four males with equal paternity.

Total execution probabilities

Single mating. As above (eq. [1]).

Quadruple mating. Scenario: random mating. The paternities of the four males are equal, 25%. The proportion of diploid males is 0%, 12.5%, 25%, 37.5%, and 50% for queens who have made zero, one, two, three, and four matched matings, respectively. The probabilities of these outcomes are given by the terms of the binomial theorem:

Box 1 (*Continued*)

p^4, $4p^3(1-p)$, $6p^2(1-p)^2$, $4p(1-p)^3$, and $(1-p)^4$. Paralleling equation (2), the total mortality of queens mated to four males is

$$M_{4\text{equal}} = m_0 p^4 + m_{0.125}\,[4p^3(1-p)] + m_{0.25}\,[6p^2(1-p)^2] + m_{0.375}\,[4p(1-p)^3] + m_{0.5}[(1-p)^4]. \quad (5)$$

Mating with four males is favored if

$$m_{0.5}p > m_0 p^4 + m_{0.125}\,[4p^3(1-p)] + m_{0.25}\,[6p^2(1-p)^2] + m_{0.375}\,[4p(1-p)^3] + m_{0.5}\,[(1-p)^4]. \quad (6)$$

Where $m_0 = 0$, $m_{0.125} = 0.07$, $m_{0.25} = 0.75$, $m_{0.375} = 0.76$, and $m_{0.5} = 0.77$ (m_x values are taken from fig. 2 except $m_{0.375}$, which is interpolated between $m_{0.25}$ and $m_{0.5}$). When inequality (6) is evaluated for $p = 0.081$ (the probability of matched mating in the study population of *Scaptotrigona depilis*) it is found to be true ($0.062 > 0.044$), showing that multiple mating is favored as it reduces by approximately 29% the total probability that a queen will be executed. However, unlike single versus double mating, this result also depends on p, the probability of a matched mating in the population. As p increases, the total mortality of queens mated to four males increases more rapidly than that of queens mated to a single male, and at a value of $p = 0.17$, approximately, queen survival (nonexecution) is equal (fig. 3). The reason is that when p increases, the magnitudes of the higher-order terms in p, especially p^2, which generate enough diploid males to result in queen execution, are increased.

In principle, a sigmoid function could be fitted to the queen execution data in figure 2 and used to calculate the total survival of queens of any mating frequency and any pattern of paternity using the binomial theorem to determine the relevant frequencies of the different outcomes in terms of the proportion of diploid males. In practice, it is premature to do this, as much additional data would be needed to fit the function in the middle part, between 12.5% and 25% diploid males, where the execution probability changes rapidly. The text provides a discussion of some special cases of multiple mating with unequal paternities.

or a break in brood cell production, which indicates lack of an egg-laying queen.

Results

Figure 2 shows the survival of the 70 queens in the four groups of test hives. As expected, queen mortality was high in colonies producing 50% (77% [10/13]) diploid males and low in colonies producing 0% diploid males (0% [0/11]; $P = .0002$, Fisher's exact test [FET], two-tailed). Queen mortality in colonies producing 25% diploid males was also high (75% [24/32]), very similar to that in the colonies producing 50% diploid males ($P = 1$; FET, two-tailed). Queen mortality in colonies producing 12.5% diploid males was low (7% [1/14]), very similar to that in the colonies producing 0% diploid males ($P = 1$; FET, two-tailed). Queen mortality in colonies producing 12.5% diploid males was significantly lower than that in colonies producing 25% diploid males ($P < .0001$; FET, two-tailed). Data underlying figure 2 have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.88h3gp6; Vollet-Neto et al. 2019).

Our data are insufficient to determine the exact shape of the sigmoid relationship between queen mortality and the proportion of diploid males. However, the data clearly show two important results and confirm that this relationship has a sigmoid shape. First, below a certain threshold proportion of diploid males—approximately 12.5%—queen mortality is low, similar to 0% diploid males. Second, above a certain threshold—approximately 25%—queen mortality reaches a maximum similar to 50% diploid males. These results are sufficient for testing the queen execution hypothesis.

Although the mortality of our queens in colonies with 50% diploid males is slightly lower than in a previous study (10/13 [this study] vs. 20/20 [Vollet-Neto et al. 2017]), the difference is not significant ($P = .0524$; FET, two-tailed). The difference may have come about as the two studies used different methodologies and manipulations as they addressed different questions. In Vollet-Neto et al. (2017), queens that had not made a matched mating were introduced into colonies with 50% diploid male production from which the matched-mated queen had been removed and the brood combs were not manipulated. In the current study, brood combs were introduced into colonies with a queen that had not made a matched mating and who was not removed from her colony, and the brood combs were cut and manipulated, causing a reduction in the number of emerging bees per time. It is important to note that in the current study we used a design (fig. 1) that resulted in identical experimental manipulations across all test colonies so that differences in manipulation could not have caused the

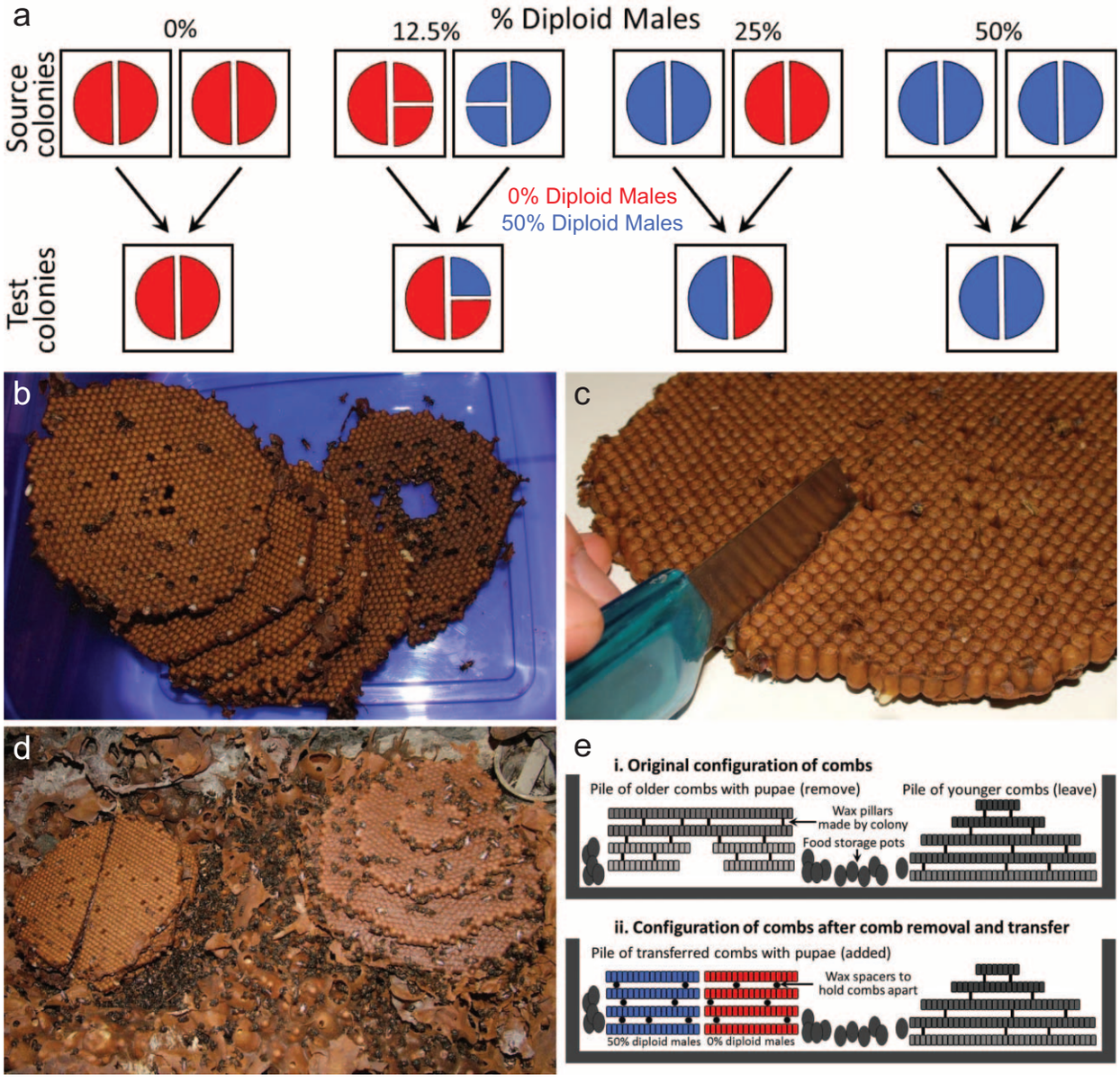

Figure 1: Setup of test colonies with different proportions of emerging diploid males. *a*, All test colonies received brood from two source colonies. The two source colonies per test colony were matched for their amounts of brood to facilitate providing equal amounts of brood to test colonies with 0%, 25%, and 50% diploid males. For test colonies with 12.5% diploid males, the area of brood with 50% diploid males was one-third that for brood containing 0% diploid males. Only older combs comprising only pupae, in their silk cocoons, were transferred. Combs containing larvae are too fragile for handling. As the brood period in *Scaptotrigona depilis* is approximately 36 days, we transferred brood every 18 days (i.e., half the brood period). Source colonies generally provided four to six combs per transfer. *b*, Six combs of pupae taken from a single source colony for transfer to test colonies. The lowest comb is the oldest, and the pupae in the center, from the oldest cells, have already emerged to leave a central hole. *c*, Cutting a brood comb in half. The pupae are in cocoons with the wax that formed the cells already removed by the worker bees. *d*, Test hive showing two brood piles, as occurs normally in *S. depilis* colonies. The pile on the left is composed of old brood transferred in from the source colonies; the pile on the right is young brood produced in the colony. Many spherical food-storage pots and bees can also be seen. *e*, Schematic (not to scale) of *d* from the side showing how the left-hand pile of older brood combs had been removed and replaced with a brood pile composed of comb halves taken from two source colonies (i); these were introduced as a group and were separated vertically using spacers (small balls of wax taken from the colony) to duplicate the natural situation in which the worker bees build wax pillars to provide supports and access space between adjacent combs (ii).

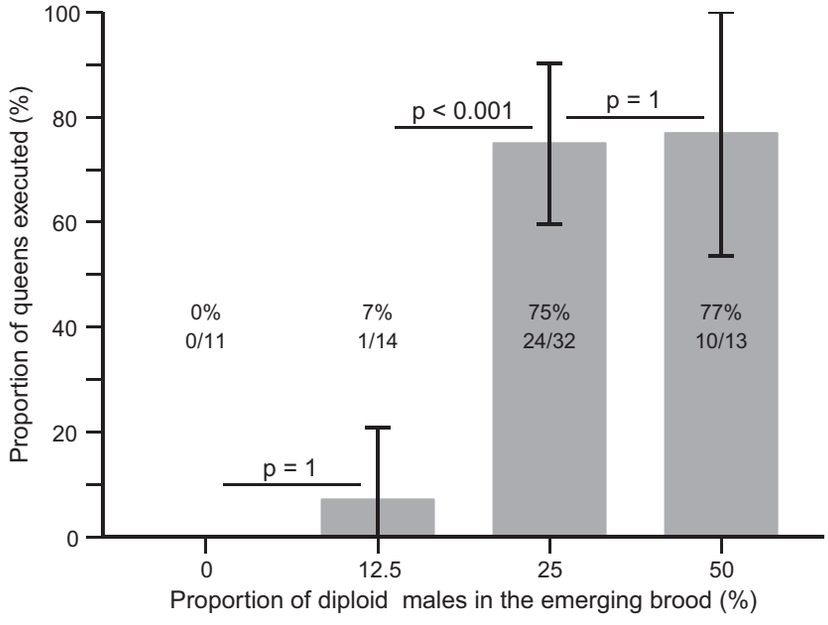

Figure 2: Proportion of queens eliminated in the test colonies ($n = 70$) with different experimental proportions of diploid males in the emerging diploid brood. *P* values are from 2 × 2 contingency tests using Fisher's exact test (two-tailed). Numerators show the number of queens executed, and denominators show the number of queens tested. The error bars show the 95% confidence interval.

variation in queen mortality that occurred among the four test groups.

Discussion

Our results support the queen execution hypothesis for single mating by queen stingless bees. The results indicate that *Scaptotrigona depilis* queens that mate with two males and use the sperm equally will have higher total mortality than queens that mate with only one male. In particular, the results show that queen execution probability is almost identical for queens heading colonies with 25% (one matched mating of two matings with equal sperm use) versus 50% (one matched mating of one mating) diploid males (fig. 2). However, when a queen mates with two males, this approximately doubles her chance of making a matched mating (see box 1). Overall, therefore, double mating will cause increased queen mortality and select for single mating (box 1).

Our additional results shows that the mortality of queens heading colonies with 12.5% diploid males is almost identical to that of queens heading colonies with 0% diploid males (7% vs. 0%; fig. 3). This has two important additional implications for the possible evolution of the mating system away from monandry and toward polyandry.

Although our data indicate that double mating with equal sperm use will be selected against, the additional queen mortality data from test colonies producing 12.5% diploid males indicate that double mating with unequal sperm use will be selectively neutral. In particular, the results show that if the minority male contributes a small proportion of the paternity—less than approximately 25%—then this will not result in increased total queen execution probability. When a queen mates with two males, one of whom is a matched mating, then with a probability of one-half the matched male will be the minority male and so will not trigger execution, thus resulting in no additional mortality for double-mated queens (box 1). Our data indicate that this nonexecution threshold is close to 12.5% diploid males, equivalent to 25%:75% paternity shares of two males. Conversely, when the majority male is a matched mating this will lead to execution, in a similar way to a monandrous matched mating.

Interestingly, analysis of progeny genotypes with DNA microsatellite markers indicates that some *S. depilis* queens do mate with two males but have highly unequal paternity (Paxton et al. 2003). A second patriline was detected in the progeny of two out of 10 queens studied, with the minority males contributing 1 of 20 (5%) and 1 of 16 (6%) of the worker progeny analyzed. Our results indicate that this situation, in which the effective paternity frequency of double-mated queens is close to 1, will be selectively neutral with respect to queen execution. Paternity skew in the progeny of multiple-mated queen Hymenoptera is, in general, higher in species with low paternity frequency (Jaffé et al. 2012). In the case of double mating, double-mated queens in four species of *Dolichovespula* wasps, which are usually monandrous, had highly unequal paternity (Foster et al. 2001). By contrast,

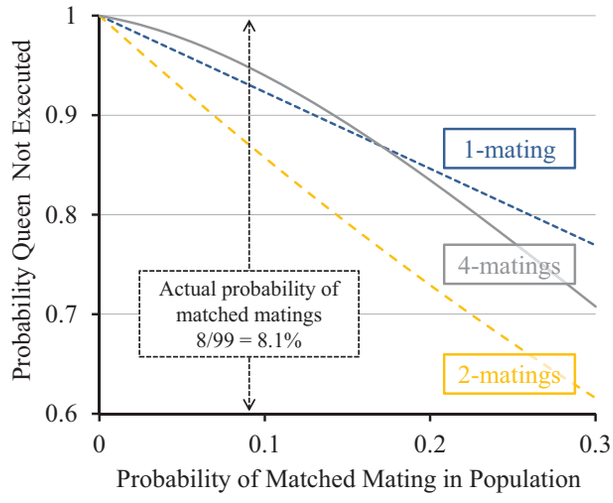

Figure 3: Probability that a queen who has mated with one, two, or four males with equal paternity will not be executed due to diploid male production in her colony as a function of the probability of making a matched mating in the population. The proportion of queens in the study area that made matched matings is 0.0808 ($n = 8/99$).

double-mated queens in *Vespula vulgaris*, a species which is usually polyandrous, showed paternity shares that were not significantly different from equal (Foster and Ratnieks 2001).

Queen mortality data from colonies with 12.5% diploid males also indicate that polyandry will be selected for if paternities are equal (box 1, pt. B). If a queen mates with four males and uses sperm equally, then a single matched mating will result in 12.5% diploid males and will result in low or zero queen execution. In some cases a polyandrous queen will make more than one matched mating. However, the probability of making two or more matched matings out of four is low when the probability of making a matched mating is low. The probability of making more than one matched mating increases as the probability of making a matched mating increases in the population (box 1). This means that when the probability of making matched matings is low, the execution probability of queens mated to four males is lower than that for single-mated queens (fig. 3).

It is very likely that the probability of making matched matings is sufficiently low to cause the execution of queens mated to four males to be lower than that for single-mated queens. This is because frequency-dependent selection favors rare alleles at the CSD locus and so generates high allelic diversity (Yokoyama and Nei 1979; Cook and Crozier 1995; Gloag et al. 2016), as observed for several populations of eusocial bees (Adams et al. 1977; Alves et al. 2011; Francini et al. 2012). For our population, the probability of making a matched mating was 8.1%. As a result, the probability of making two or more matched matings for queens mated to four males, which will result in 25% or more diploid males and a high probability of queen execution, is only 3.5%. By contrast, with double mating the chance of making one matched mating and so having 25% diploid males is 15%, versus 8% for single-mated queens. For our study population, the data indicate that queens mating to four males would have a lower probability of being executed than queens mated to one male, as the probability of making a matched mating is <0.17 (box 1; fig. 3). However, in small populations the probability of making a matched mating can be higher. Zayed and Packer (2001) estimated an effective population size of only 19.6 in a species of bee, *Halictus poeyi*, with the probability of matched matings estimated to be 18%–100%.

Therefore, the effect of queen execution on selecting for or against polyandry will depend both on how unequal the paternity shares are and on the probability of making a matched mating. In the case of mating with four males in a population in which the probability of matched matings is low, the most relevant potential outcomes include the following.

Outcome 1: Very low paternity in all but one male; polyandry selectively neutral (equivalent to single mating). If all but one male has a low share of paternity (e.g., 85%, 5%, 5%, 5%), then this will be similar to single mating. This is because a matched mating will cause queen mortality only if it is by the majority male. Matched matings in the minority males will have no effect, even if all of them have made a matched mating, as this will only result in a small proportion of diploid males in the colony (2.5%–7.5% in the example) and be below the queen execution threshold.

Outcome 2: Low paternity in all but one male; weak selection against polyandry (nearly equivalent to single mating). All but one male has a low share of paternity (e.g., 50%, 16.7%, 16.7%, 16.7%). The majority male has enough paternity to cause queen execution if he is a matched mating. The minority males have insufficient paternity to cause queen execution if only one is a matched mating, but if more than one of them are matched matings this will exceed the execution threshold. Selection against polyandry will occur but be weak, as the probability that two or more minority males make matched matings is low.

Outcome 3: High paternity in two males; selection against polyandry (equivalent to double mating with equal sperm use). If two of the males each have sufficiently high paternity to individually cause queen execution if one of them is a matched mating (e.g., 48%, 48%, 2%, 2%), then this will be similar to double mating and will cause greater total queen mortality.

Outcome 4: Equal paternity in all males; selection for polyandry. If a queen mates with multiple males that have equal or near equal paternity (e.g., 25%, 25%, 25%, 25%), then a single matched mating will not exceed the execution threshold and the total probability of queen execution will reduce

in comparison to monandry (box 1). Although two or more of a queen's mates may make matched matings, resulting in queen execution, the chance of this occurring is low when the probability of making a matched mating is low. For example, the probabilities of making zero, one, two, three, and four matched matings, for a queen that mates with four males, are, respectively, p^4, $4p^3(1-p)$, $6p^2(1-p)^2$, $4p(1-p)^3$, and $(1-p)^4$, where p is the probability of making a matched mating. For $p = 0.08$, these values are 0.7139, 0.2510, 0.0331, 0.0019, and <0.0001, respectively. That is, most matched matings do not trigger queen execution, as they occur in only one of a queen's mates not in two or more.

Overall, our results suggest that queen execution due to matched matings and diploid male production results in a fitness landscape (fig. 4) with two adaptive peaks: (1) monandry or multiple mating with unequal sperm use such that the effective paternity is close to 1 and (2) polyandry in which queens mate with four or more males and use sperm equally. Intriguingly, our analysis indicates that it is not possible to evolve gradually from the monandry peak to the polyandry peak, for example, with queen mating frequency passing through stages of 1 (monandry) → 1.1 (double mating with unequal paternity) → 2 (double mating with equal paternity) → 4 (quadruple mating with equal sperm use). If sperm use is equal, then double mating is selected against. Double mating is neutral, with respect to queen execution, when sperm use is unequal. However, if sperm use under double mating evolves to be more equal than approximately 25%:75%, it will be selected against. Could queen mating frequency evolve directly from monandry to polyandry? If so, the low-fitness valley of double mating could be jumped over.

There are many other costs and benefits not related to the diploid male load that may also effect the evolution of queen mating frequency in eusocial Hymenoptera and that may also be relevant to stingless bees (Ratnieks 1990; Pamilo et al. 1994; Tarpy and Page 2000). Our results and analysis indicate that diploid males can influence the evolution of the mating system in *S. depilis*. We hypothesize that the apparent ubiquity of monandry in stingless bees (Vollet-Neto et al. 2018) has the same cause. This is because all stingless bees rear brood in sealed cells on a provision mass (Michener

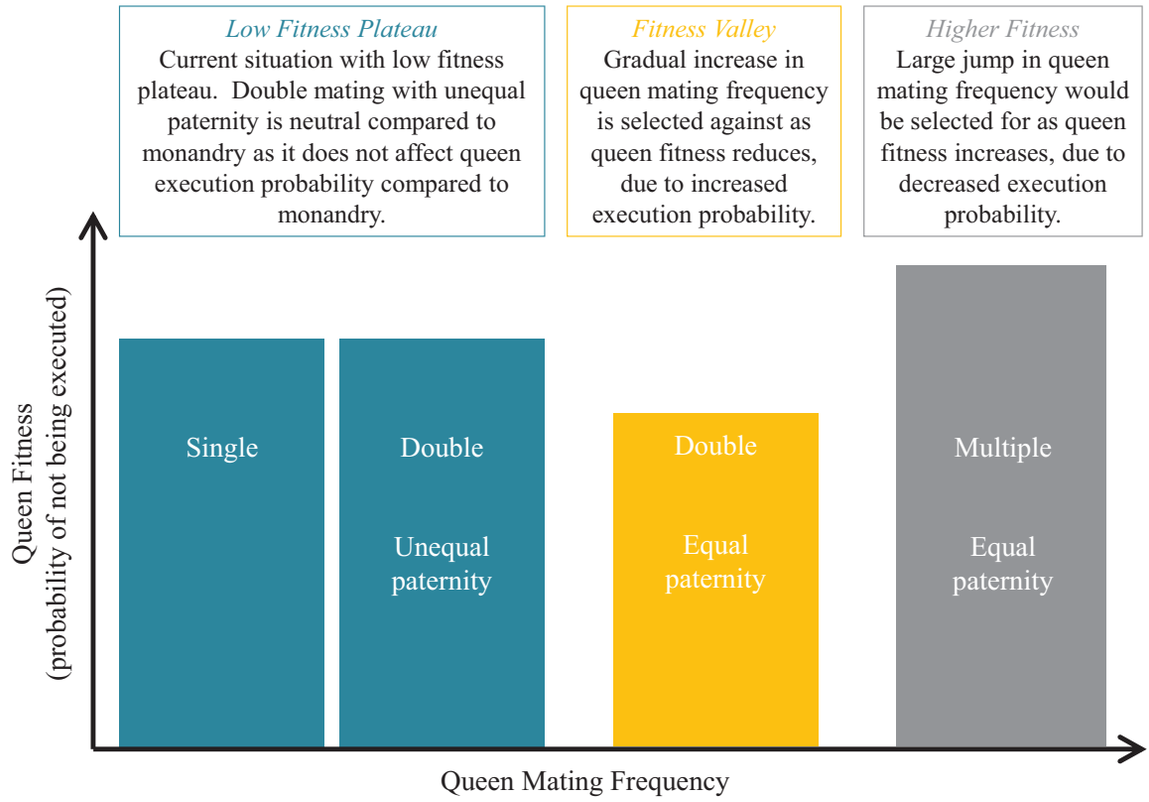

Figure 4: Hypothesized fitness landscape for queen mating frequency in *Scaptotrigona depilis* and other stingless bees, Meliponini, due to queen execution. The current situation of single mating and occasional double mating with unequal paternity (two left bars) is an adaptive peak. A second, higher adaptive peak of multiple mating cannot be accessed via gradual evolutionary increase in queen mating frequency because double mating with equal paternity has lower fitness than single mating or double mating with unequal paternity.

1974), which results in diploid males causing a high cost at the colony level, since each diploid male is reared to adulthood (Ratnieks 1990). Queen execution is known to occur in two other stingless bees, both *Melipona* (Camargo 1976; Alves et al. 2011). Diploid males also occur in other eusocial Hymenoptera. In contrast to the stingless bees, in the honey bee *Apis mellifera* there is evidence that the colony-level costs of diploid male production favor multiple mating (Page 1980; Ratnieks 1990; Cook and Crozier 1995). The reason for this difference is that honey bees rear brood in open cells and remove diploid males as young larvae before much time or energy has been invested in them (Ratnieks 1990).

Our study shows that in terms of the evolution of the mating system, the question is not just "why polyandry?" but also "why monandry?" It is also an example of the importance of an idiosyncratic difference in biology (Beekman and Ratnieks 2003), with the difference in the brood-rearing method of honey bees versus stingless bees having far-reaching and contrasting consequences in other areas of social life, including mating system (this study), reproductive conflict outcome (Bourke and Ratnieks 1999; Ratnieks et al. 2006), and disease control (al Toufailia et al. 2018).

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Dedication

We dedicate this article to the late Dr. Paulo Nogueira-Neto (1922–2019), secretary of the environment for the Brazilian government (1974–1986); member of the United Nations Brundtland Commission on the Environment and Development (1984–1987); professor at the University of São Paulo (1980–1992); conservationist, biologist, and humanitarian; student, advocate, and enthusiast of stingless bees; and stingless bee beekeeper.

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